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TAXOL INDUCES CELL DEATH WITH CYTOPLASM VACUOLIZATION IN PARAPTOSIS-LIKE BUT NOT ONCOSIS FASHION IN ASTC-a-1 CELLS

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Recently, we found that high concentration of taxol (70 μM) induced cell death with cytoplasm vacuolization, the typical characteristic of both paraptosis and oncosis, in human lung carcinoma (ASTC-a-1) cells. This report was designed to further determine the form of taxol-induced cell death with cytoplasm vacuolization. It is generally considered that the cytoplasm vacuolization in oncosis due to the swelling of endoplasmic reticulum (ER), mitochondria, lysosomes and nuclei occurs after the loss of mitochondrial membrane potential ($\Delta\Psi m$). However, flow cytometry (FCM) analysis showed that taxol-induced cytoplasm vacuolization preceded the loss of $\Delta\Psi m$. Moreover, taxol treatment did not induce the collapse of microtubule, the typical characteristic of oncosis. These data demonstrated that taxol-induced cell death with cytoplasm vacuolization is not oncosis. FCM analysis by Annexin V-FITC/PI apoptosis detection kit further demonstrated that taxol-induced cell death with cytoplasm vacuolization is not apoptosis. In conclusion, in combination with our recent *in vitro* and *in vivo* data, this report further demonstrates that high concentration of taxol induces cell death with cytoplasm vacuolization in paraptosis-like but not oncosis fashion.

Keywords: Taxol; human lung adenocarcinoma cancer cells; paraptosis; oncosis; microtubules; endoplasmic reticulum.

1. Introduction

Cell death has been classified into the two large categories: programmed cell death (PCD) and necrosis. Apoptosis is the best characterized form of PCD with membrane bleb, loss of the asymmetry of phosphatidylserine (PS) in the plasma membrane, nuclear fragmentation and activation of caspases.[1] In contrast to apoptosis, necrosis is a less-ordered event resulting from cell injury and is commonly associated with inflammation. Necrosis does not produce any regular pattern of DNA or protein degradation and is characterized by cell swelling, dilatation of mitochondria, increase of plasma membrane permeability and disruption of the plasma membrane.[2,3]

Oncosis is also a form of cell death accompanied by cellular swelling, organelle swelling, blebbing, increased membrane permeability, with a non-specific DNA degradation.[4,5] Moreover, oncosis has been characterized as an unorchestrated event, resulting in bursting.[6] Although it has long been confused with necrosis and oncosis processes leading to cell death, necrosis refers to the morphological alterations appearing after either apoptosis or oncosis. Necrosis is defined by the loss of plasma membrane integrity and apoptosis is characterized by cellular shrinkage, nuclear condensation, and activation of caspase-3,[7,8] whereas oncosis is marked by intracellular swelling.[4] It is recognized that breakdown of the plasma membrane and down-regulation of the cytoskeleton-associated proteins such as talin, paxillin and vinculin are associated with oncosis.[9] Loo *et al.*[10] also demonstrated that RAV12 caused oncosis by inducing disruption of cytoskeleton. Moreover, in oncosis, cellular energy depletion following metabolic insults such as severe ischemia produces dramatic reduction in mitochondrial respiration and ATP synthesis, resulting in a loss of ionic homeostasis and cellular swelling.[4] Because the mitochondrial generation of ATP requires electrochemical gradient, a reduction in mitochondrial membrane potential may precede the morphologic changes observed in oncotic cell death.[11]

We recently found that high concentration of taxol (70 μM) induced cell death with cytoplasm vaculization in paraptosis-like fashion in HeLa, U87, A549 and ASTC-a-1 cell lines,[12–14] and low concentration of taxol (35 nM) induced cell death mainly in apoptotic fashion.[15] Cytoplasm vacuolization from endoplasmic reticulum (ER) and/or mitochondrial swelling together with absence of apoptotic DNA fragmentation and caspase activation are the characteristics of paraptosis.[16,17] However, the cytoplasm vacuolization is also the typical characteristic of oncosis in morphology.[4] Compared with paraptosis, oncosis is also characterized by ER swelling and caspase inactivation as well as mitochondrial swelling.[18]

This report is designed to further determine the fashion of taxol-induced cell death with cytoplasm vacuolization by assessing the temporal relation between cytoplasm vacuolization and dissipation of the mitochondrial membrane potential ($\Delta\Psi m$), and the collapse of microtubule cytoskeleton in ASTC-a-1 cells. Our data further demonstrate that taxol-induced cell death with cytoplasm vacuolization is paraptosis-like but not oncosis.

2. Materials and Methods

2.1. *Materials*

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY). Taxol was purchased from Haikou Pharmaceutical Co. Ltd. (Haikou, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Annexin V-FITC/prodium iodide (PI) apoptosis detection kit was obtained from Bender Medsystems (Vienna, Austria). Staurosporine (STS) was purchased from Alexis (Lausen, Switzerland).

2.2. *Cell culture and transfection*

ASTC-a-1 cell line obtained from the Department of Medicine, Jinan University (Guangzhou, China), was cultured in DMEM (Gibco, Grand Island, USA) supplemented with 10% fetal calf serum. Cell cultures were maintained at 37°C in a humidified 5% CO_2 incubator. Plasmids DNA of enhanced green fluorescent protein (EGFP) microtubule were transiently transfected into the cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, USA).

2.3. *Apoptosis*

Cell apoptosis detection was performed by flow cytometry (FCM, FACSCanto II, BD, New Jersey, USA) analysis using Annexin V-FITC/PI apoptosis detection kit (Bender Medsystems, Vienna,

Austria) and 10,000 events were recorded for each FCM analysis.

2.4. *Monitoring microtubule distribution inside living cells by confocal microscope imaging*

Microtubule distribution was assessed using confocal fluorescence microscopy (LSM510, Zeiss, Jena, Germany). Cells transfected with EGFP microtubules were treated with 70 μM taxol for 10 h. All the quantitative analysis of the fluorescence images was performed by Zeiss Rel3.2 image processing software (Zeiss, Jena, Germany). EGFP was excited at 488 nm and fluorescence emission was recorded through a 500–550 nm band-pass filter.

2.5. *Measurement of mitochondrial membrane potential ($\Delta\Psi m$)*

Rhodamine 123 (Rho 123, Sigma, St. Louis, USA) was used to analyze $\Delta\Psi m$ by FCM as previously described.[12] Briefly, cells were harvested and stained with 10 μM Rho 123 for 30 min at 37°C in the dark, and then washed with phosphate buffer solution (PBS) twice and subsequently assayed by FCM. Results were expressed as the proportion of cells with low Rho123 fluorescence indicating the loss of $\Delta\Psi m$.

3. Results

3.1. *Taxol-induced cytoplasm vacuolization precedes the dissipation of $\Delta\Psi m$*

Our previous *in vitro* data have shown that taxol can induce significant induction of cytoplasm vacuolization.[12–14] In this study, 70 μM of taxol was adopted in all experiments as described previously.[12,13] We used FCM analysis to assess the temporal relationship between the taxol-induced cytoplasm vacuolization and dissipation of $\Delta\Psi m$. As shown in Fig. 1, percentage of the cells showing side scatter (SSC) indicative of the complexity of cellular organelles has a big increase from (4.4%) (control) to 16.1% at 3 h after taxol treatment, but percentage of the cells with low $\Delta\Psi m$ was almost constant until 3 h after taxol treatment, indicating that taxol-induced vacuolization preceded the loss of $\Delta\Psi m$.

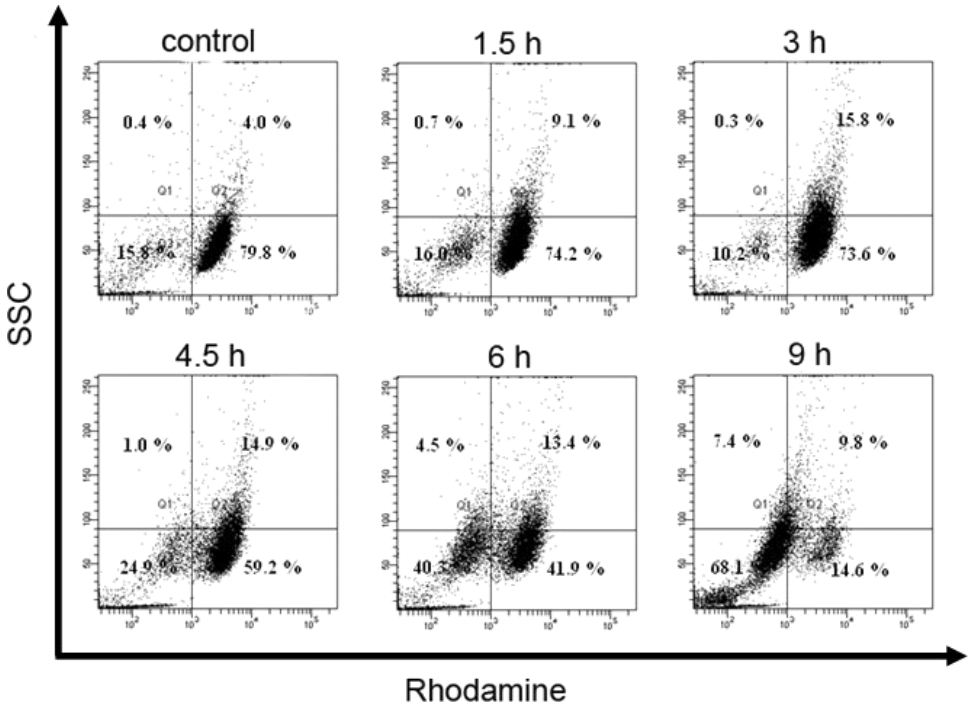

Fig. 1. Taxol-induced cytoplasmic vacuolization precedes the dissipation of $\Delta\Psi m$. The cells treated by taxol were stained with Rhodamine 123 for 30 min, and the fluorescence intensity was detected by FCM to analyze the loss of mitochondrial membrane potential ($\Delta\Psi m$).

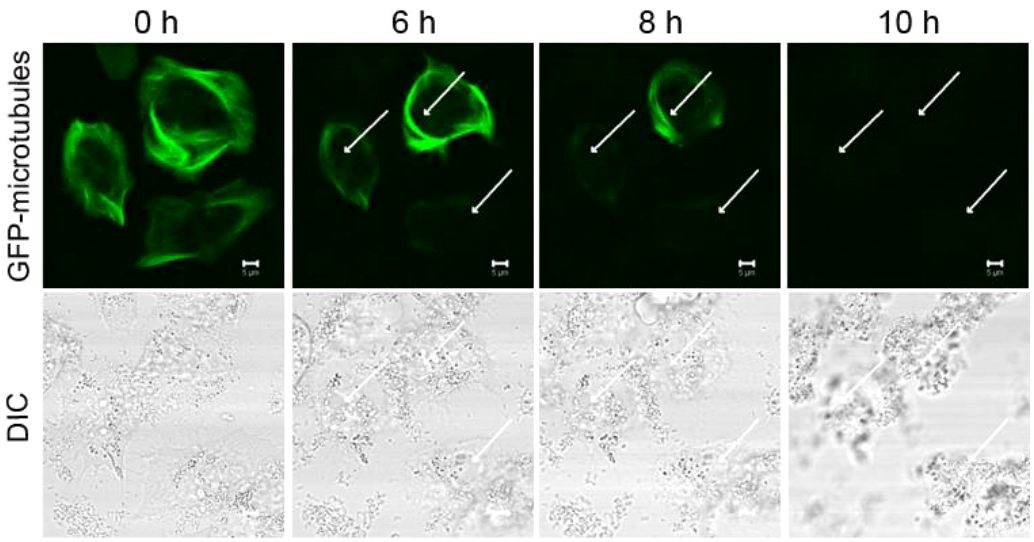

Fig. 2. Dynamical confocal imaging of living cells showing the cytoplasm vacuolization and the distribution of microtubules after taxol treatment. White arrows indicate the taxol-induced cytoplasm vacuolization. Scaled bar: 5 μm.

3.2. *Taxol does not induce the collapse of microtubule cytoskeleton*

Collapse of the microtubule cytoskeleton is the typical characteristic of oncosis.[19] To determine whether taxol-induced cell death belongs to oncosis, we used confocal imaging to monitor the dynamics of microtubule cytoskeleton during taxol-induced cytoplasm vacuolization in a single live cell. As

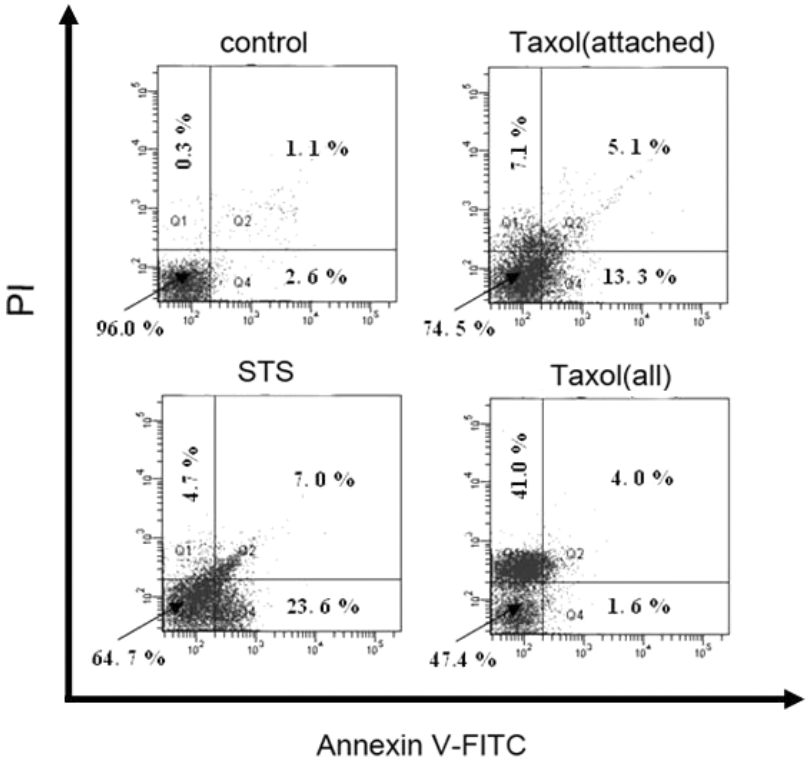

Fig. 3. Taxol-induced nonapoptotic cell death. Cells were pretreated with 70 μM taxol for 18 h, and then were treated with Annexin V/PI double staining method for apoptosis detection. *Taxol* (*attached*), attached cells after taxol treatment; *Taxol* (*all*), attached and suspended cells after taxol treatment.

shown in Fig. 2, taxol treatment for 6 h induced cytoplasm vacuolization (white arrow), but exposure of cells transfected with EGFP-microtubule plasmids to taxol for 8 h did not induce the collapse of microtubule cytoskeleton, indicating that microtubule did not participate in the taxol-induced cytoplasm vacuolization.

3.3. *Taxol induces nonapoptotic cell death*

We previously used confocal microscopic imaging to assess the membrane integrity of cells, and demonstrated that taxol induced cell death with cytoplasm vacuolization in non-necrotic fashion.[12,13] In order to further determine whether taxol induces cell death in apoptotic fashion, we used FCM to analyze the externalization of PS and membrane integrity of cells co-stained by Annexin V/PI. As shown in Fig. 3, the attached cells after taxol treatment for 18 h showed a slight increase in the percentage of cells with Annexin V^+, while all of the taxol-treated cells (including the attached and suspended cells) did not show a marked increase in the percentage of cells with Annexin V^+, indicating that taxol induced cell death in nonapoptotic fashion. The final outburst of the taxol-treated cells with cytoplasm vacuolization led to a prominent increase of the cells with Annexin V^-/PI^+ (see Fig. 3). STS (1 μM)-treated cells were used as positive control.

Taken together, the present data suggest that taxol-induced cell death is similar to paraptosis but not oncosis.

4. Discussion

Our *in vivo* and *in vitro* data have demonstrated that high concentration of taxol can induce cell death with cytoplasm vacuolization, the typical characteristics of both paraptosis and oncosis. We referred to this cell death form as parapotis-like cell death according to the similarity in morphology.[12–14] We have demonstrated that the taxol-induced vacuolization is from ER and mitochondrial; we have further demonstrated that caspases and Bcl-XL do not participate in the taxol-induced cytoplasm vacuolization. However, we have not delineated the taxol-induced cell death as paraptosis-like cell death or oncosis previously.[14] Here, we found that taxol-induced vacuolization precede the taxol-induced collapse of mitochondria, and taxol did not induce the collapse of microtubule cytoskeleton, indicating that taxol-induced paraptosis-like cell death is not oncosis.

Our previous data that cells maintain the integrality of plasma membrane even at 11 h after taxol treatment[13] demonstrate that microtubules sustain the ability to support the plasma membrane. Our results indicate that taxol-induced cell death with cytoplasm vacuolization is not oncosis. Oncosis is a rapid process characterized by vacuolization, mitochondrial swelling and loss of plasma membrane integrity.[4] Oncosis generally occurs in seconds to minutes at most 1 h and the lysis of cells can be observed in short time.[9,10,19] Our dynamic observation that taxol does not induce the collapse of microtubules cytoskeleton after cytoplasm vacuolization (see Fig. 2) further demonstrated that taxol-induced cell death with cytoplasm vacuolization is not oncosis.

Moreover, the facts that taxol-induced cytoplasm vacuolization precedes the loss of mitochondrial membrane potential ($\Delta\Psi m$) further demonstrate that taxol-induced cell death is not oncosis. Cellular ATP level plays a pivotal role in oncosis. If the mitochondrial damage causes rapid and excessive ATP depletion, the cell goes to oncosis.[20,21] Kyoi *et al.*[22] reported that 2,4-dinitrophenol (DNP) — an uncoupler of mitochondrial respiration, or oligomycin, an inhibitor of mitochondrial F1F0-ATPase-induced oncosis, even the cells were pretreated by ischemic preconditioning protecting mitochondria, implying that oncosis is the consequence of ATP depletion. ATP depletion may induce mitochondrial damage, subsequently resulting in vacuolization and oncosis.[21] However, our observations showed that the percentage of cells with cytoplasm vacuolization significantly increased at 1.5 h after taxol treatment (see Fig. 1),[13] while the percentage of cells with low $\Delta\Psi m$ increased largely until 4.5 h after taxol treatment (see Fig. 1), demonstrating that taxol-induced cytoplasm vacuolization preceded the loss of mitochondrial membrane potential ($\Delta\Psi m$). These data also showed that taxol-induced cell death is not oncosis.

Our finding that taxol treatment does not induce a big increase of PS externalization further demonstrates that the form of cell death induced by taxol treatment is not apoptosis. Although the attached cells showed a marked increase of the

percentage of cells with PS externalization, FCM analysis for all of the cells (including attached and suspended cells) did not show a marked induction of the cells with PS externalization (see Fig. 3), demonstrating that taxol induced cell death dominantly via a nonapoptotic pathway. Confocal imaging of cells showed that the attached cells treated with taxol for 18 h were not stained by PI.[13] However, FCM analysis showed a marked increase of cells with PI staining after taxol treatment for 18 h (see Fig. 3). This contradiction may be due to the burst of the taxol-induced vacuolized cells. Our dynamical confocal imaging showed that the attached vacuolized cells after taxol treatment bursted once suspended (data not shown). In addition, the pretreatment before FCM analysis may also increase the percentage of cells with loss of membrane integrity.

In summary, in combination with our previous studies, the present data further demonstrate that high concentration of taxol induces tumor cell death with cytoplasm vacuolization via paraptosis-like but not oncosis fashion.

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